RIPENING-RELATED PERTURBATIONS IN APPLE CELL WALL NUCLEAR SPIN DYNAMICS

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Key Word Index—*Malus pumila*; Rosaceae; apple fruit; ^{1}H, $^{23}Na^{+}$ spin-lattice and ^{1}H, ^{13}C rotating frame spin-lattice relaxation times; molecular motion; polyuronides; polygalacturonase II.

Abstract—Spin-lattice ($^{1}HT_1$, $^{23}Na^{+}$ T_1) and rotating frame spin-lattice ($^{1}HT_{1\rho}$, $^{13}CT_{1\rho}$) relaxation times were measured on intact, critical point dried apple tissue at various degrees of ripeness using cross polarization and magic angle spinning (CPMAS) NMR techniques. Solid state carbonyl (δ172) $^{13}CT_{1\rho}$ and $^{23}Na^{+}$-carboxylate anion T_1 values, which are inversely proportional to carboxylate reorientation rates, decreased 15–19% during the time course study. Carbonyl resonance $^{1}HT_1$s diminished by 63% as the tissue softened; a maximal decline of 42% was also observed in the $^{1}HT_1$s of nonspecific carbohydrate ring carbon signals (δ74) indicating an increase in both acidic and neutral polymer motion. Treatment of the cell wall with polygalacturonase resulted in a significant decrease in both carbonyl and ring carbon $^{1}HT_1$s (57 and 42%, respectively) demonstrating the important structural function of polyuronides not only in the middle lamellae but also in the primary cell wall.

INTRODUCTION

Polyuronides are a major component of the middle lamellae of plant cells and are also detected in the primary cell wall [1]. In most fruits, the softening process is associated with increasing hydrolytic enzyme activity, particularly endopolygalacturonase (endoPG) [2–15]. Apples are unusual since no endoPG activity has been demonstrated [16]. Although exopolygalacturonase (exoPG) is present, this enzyme can account for only a 10% loss of uronic acids during ripening [17]. Since the physical properties [18] of water-soluble pectin from ripe and unripe fruit are similar, these substances are unlikely to be degradation products [9] of a polyuronide degrading enzyme. Other workers [16, 19] have suggested that softening in apples involves some process other than polyuronide degradation.

Cross polarization and magic angle spinning (CPMAS) NMR spectroscopy is one of the few methods available for studying the dynamic properties of bulk materials [20] such as cell walls. In particular, ^{13}C CPMAS is a high resolution solid state NMR method which allows both the examination of specific resonances associated with structural features, and the relaxation times or spin dynamics of these structural domains. Adequate reviews of this subject have been published [21, 22].

Many of the mechanical properties of polymeric materials can be ultimately related to polymer chain dynamics and localized molecular motions [20–22]. Because of the lack of good understanding of the physical properties of plant cell wall fiber interactions, and due to the questions surrounding polyuronide degradation in certain pome fruits, we report here relaxation time studies of intact apple fruit cell wall polymers as a function of ripeness and specific enzymatic degradation.

RESULTS AND DISCUSSION

The primary focus of our work is the characterization of localized molecular motions of uronide-containing polymers in intact cell walls during fruit ripening. We have studied these processes by the determination of various spin-lattice relaxation times, which are, in solids, inversely proportional to molecular motion [20–23]. The major functionalities observed in the ^{13}C CPMAS spectrum of cell wall material are the anomeric (δ105) and nonspecific carbohydrate ring carbon (δ74) resonances. These signals are not specific to a particular cell wall component, but result from the combined resonances of all carbohydrate ring carbons. Thus, relaxation times determined from these signals are measures of polymer reorientation rates or motions averaged across all cell wall components. However, relaxation times measured from the carbonyl resonance (δ172) are inversely proportional to the motion of either carboxylate groups specifically, as measured by carbonyl $^{13}CT_{1\rho}$, or to the motion of whole uronide-containing polymers, as measured by $^{1}HT_{1\rho}$ and $^{1}HT_1$. This latter case is true because the carbonyl signal results from the transfer of magnetization from a pool of ^{1}Hs associated with cell wall components containing carboxylate groups.

Rotating frame spin-lattice relaxation times of the carbonyl resonance ($^{13}CT_{1\rho}$, Fig. 1), which are sensitive to motions in the 10–100 kHz frequency range [20], were measured on intact, ethanol-dehydrated and critical point dried tissues obtained from apples (cv Golden Delicious) before and after 21 days of ripening at 20°. The same

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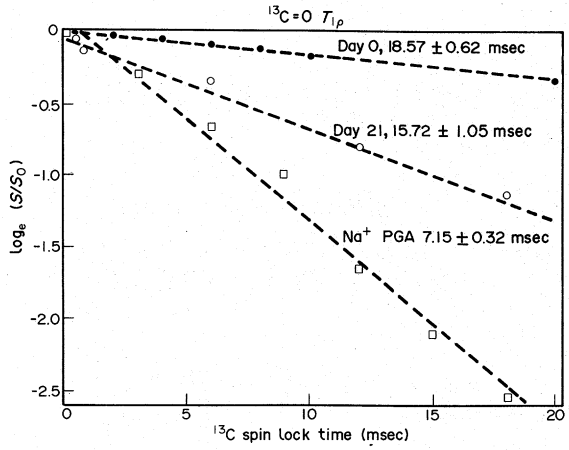

Fig. 1. Natural log of normalized carbonyl signal intensities as a function of ^{13}C spin locking time for cell wall samples from fruit ripened 0 and 21 days at 20° and for the Na^+ salt of polygalacturonate (Na^+PGA). Slope = $-(1/T_{1\rho})$. The calculated relaxation times are given ± s.e.

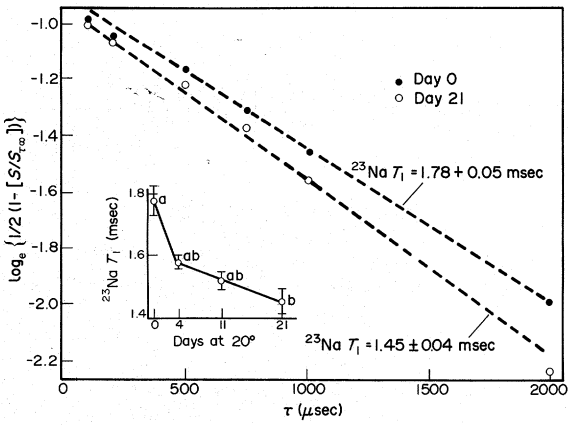

Fig. 2. Integrated 1st order rise in carboxylate-bound $^{23}Na^+$ magnetization via saturation recovery. Inset graph: plot of $^{23}Na^+$ T_1s ± s.e. as a function of ripening time at 20°. $S_{t\infty}$ = $^{23}Na^+$ signal intensity at a long delay time (50 msec) following the 90° pulse train. Slope = $-(1/T_1)$.

measurements were made on the Na^+ salt of polygalacturonate (Na^+PGA). Carbonyl ^{13}C $T_{1\rho}$ values decreased *ca* 15% during the time coincident with ripening and were 2.5–2.9-fold greater than the equivalent Na^+PGA relaxation time. These data indicate that the technique is sensitive to obvious differences in molecular size, aggregation and methylation between the ordered and amorphous states. However, carbohydrate ring carbon (δ74) ^{13}C $T_{1\rho}$s were *ca* equivalent (3.51 ± 0.14, 4.33 ± 0.28 and 3.91 ± 0.18 msec; $\bar{X}$ ± s.e. for day 0, 21 and Na^+PGA, respectively). In order to confirm these differences in cell wall carboxylate motion, $^{23}Na^+$ T_1 measurements (Fig. 2) were made on the Na^+ salts of these samples. Titration with H_2O (pH = 2) yielded 11.78 ± 0.24, 10.19 ± 0.31, 10.53 ± 0.42 and 7.41 ± 0.67 mg Na^+/g cell wall material (days 0, 4, 11 and 21, respectively) indicating that certain H_2O soluble fragments have been lost. Since polyuronides are the major anionic species in fruit tissues [13, 18] and cell wall-bound Na^+ was acid titratable, the carboxylate anion is the likely site of Na^+ binding. The trend in the changes in $^{23}Na^+$ T_1s could be followed as a function of the ripening process (Table 1). From the line width of the $^{23}Na^+$ spectra we have estimated that the $^{23}Na^+$ T_2s were *ca* 0.22 msec and, therefore, the ratios of T_1s and T_2s were in the range of 7–8. From this knowledge, we can argue that the ripening-related drop in $^{23}Na^+$ T_1s was indicative of decreasing Na^+ correlation times or increasing Na^+ motion. Thus, the changes in $^{23}Na^+$ T_1s confirm some perturbation in carboxylate motion as was seen previously (Fig. 1). These data (Figs 1 and 2) indicate that polyuronide carboxylate groups are sensitive to attenuation of pectic substance main chain mobility as evidenced by changes in carbonyl ^1H T_1s (Table 1), which were also observed to diminish linearly with cell separation or tissue firmness. Nonspecific carbohydrate ring carbon ^1H T_1s dropped 42% initially, but gradually increased thereafter. The ring carbon ^1H T_1 alterations which occur during ripening are somewhat puzzling. These data might reflect an initial perturbation in the neutral carbohydrate polymers, which are responsible for *ca* 70–80% of the ring carbon signal, due to ripening-related changes in pectic substances. Because of the diversity of polymers represented by this signal, we cannot attribute this behavior to changes in any one component. Cell wall ^1H $T_{1\rho}$ values remained essentially constant during this time course experiment, indicating that lower frequency modes of polymer reorientation [23] have not been perturbed.

Table 1. ^1H spin-lattice relaxation (T_1) and rotating frame spin-lattice ($T_{1\rho}$) relaxation times determined from carbonyl (δ172) and nonspecific carbohydrate ring carbon (δ74) resonances in intact cell wall samples from fruit ripened 0, 4, 11 and 21 days at 20°

Days	Firmness (kg)	T_1		$T_{1\rho}$ (msec)	
		δ172	δ74	δ172	δ74
0	6.6 ± 0.4*	333 ± 12	245 ± 3	4.3 ± 0.4	4.2 ± 0.2
4	5.7 ± 0.4	235 ± 51	142 ± 15	—	—
11	4.9 ± 0.4	180 ± 16	160 ± 4	—	—
21	4.5 ± 0.1	123 ± 15	207 ± 4	4.6 ± 0.5	4.1 ± 0.2

* ± s.e.

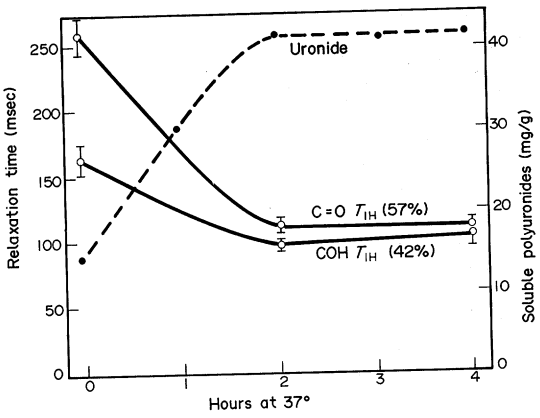

Fig. 3. Changes in carbonyl and nonspecific carbohydrate ring carbon $^1H\,T_1$s and water soluble polyuronides as a function of time at 37° in the presence of tomato polygalacturonase II.

To determine the role cell wall polyuronides play in altering carbohydrate ring carbon relaxation, day 0 wall material (Fig. 3) was treated for various times with PG II, an endo-cleaving enzyme from tomato [24]. After 2–4 hr of enzyme treatment, both carbonyl and carbohydrate ring carbon resonance $^1H\,T_1$s decrease 57 and 42%, respectively, indicating that the specific degradation of polyuronides does structurally affect the entire cell wall network.

In summary, we have observed significant changes in carboxylate group and polyuronide-containing cell wall polymer dynamics as a function of fruit ripening. The magnitude and specificity of these changes are consistent with the hypothesis that some uronide-degrading enzyme is operational during the ripening process. However, these observations could also be due to changes in the aggregation states of the constituent polymers. One hypothesis [16] proposes that ripening might be affected by changes in an active pumping mechanism which regulates the ionic status of the cell wall region. Another viewpoint [19] centers around the idea that fruit softening might be associated with an increasing degree of polyuronide carboxylate group methylation, which could also disrupt ionic bridges between adjacent uronide-containing polymers. The wall-bound Ca^{2+} concentration of these samples did not change ($444 \pm 2\ \mu g/g$). We have also determined (via ^{13}C CPMAS NMR) the relative areas of the methyl ester ($\delta 54$) and total carboxylate or carbonyl ($\delta 172$) resonances for these samples. Regardless of treatment, the degree of methyl esterification was similar ($64 \pm 5\%$). The absolute values may be in error due to nonquantitative responses of carbonyl ($\delta 172$), anomeric ($\delta 105$) and methoxy ($\delta 54$) resonances due to cross polarization. Our observations concerning the relative changes in methyl esterification, however, would not be affected. These relatively small differences in uronide structure appear to preclude the possibility of biophysical softening [16, 19] and indicate an enzymatic mechanism, perhaps the presence of an unidentified endo-cleaving enzyme. Our data (Fig. 3) also demonstrates that an intimate association exists between pectic substances and other cell wall polymers, since the decrease in size of one species affects the molecular reorientation rate of the other.

EXPERIMENTAL

Apple fruits (cv Golden Delicious) were obtained from the Beltsville Agricultural Research Center. Fruits were picked on 26 September, 1982, randomized, and immediately stored at 0° in 1% (v/v) O_2. After 1 month, *ca* 50 fruit were removed and ripened for periods of 0, 4, 11 and 21 days at 20° in a flow-through system with humidified, ethylene-scrubbed air. At each time interval, 10 fruits were randomly selected and tissue firmness measured with an Instron Universal* testing device equipped with a 0.79-cm diameter plunger attachment. The fruits were peeled, cut up into *ca* 5 × 5 × 3 mm sections and dipped in chilled 0.01 M $CaCl_2$ to inhibit tissue oxidation. The tissues were immediately vacuum infiltrated 2 hr or more in each of the EtOH–H_2O solns (consecutively in 20, 40, 60 and 80% EtOH). The fixed tissue was then equilibrated in 95% EtOH–H_2O for 1 day with repeated soln replacement. The specimens were further reduced in size to 1 mm sections and equilibrated 1 hr each in three changes of EtOH (100 ml/500 mg dry wt equivalent) followed by critical point drying. This drying technique should maintain cell wall fiber morphology, since the specimens have not been subjected to either the surface tension forces or the freezing and sublimation boundaries associated with freeze drying procedures. Dried samples were stored *in vacuo* in the presence of dehydrated silica gel. Na^+PGA was purchased from Sigma.

Na^+ *exchange.* *Ca* 2 g of fixed cell wall material from each treatment was washed × 3 in 180 ml of pH 7 Na^+-depleted H_2O and then equilibrated 24 hr in 1 M NaCl (pH = 7) at 20°. The samples were washed as before to remove unassociated Na^+, equilibrated in EtOH and critical point dried as previously explained. About three, 30-mg dry cell wall samples were equilibrated 2 hr in 10 ml of pH 2 H_2O, and washed × 2 with equivalent vols. All washes were retained for atomic absorption analysis after appropriate dilution.

PG II cell wall treatment. Day 0 fixed cell wall material (*ca* 300 mg each for 0, 1, 2, 3 and 4 hr treatments) was equilibrated overnight in 25 ml of 0.2 M NaCl (pH = 4.5) at 37°. The solns were decanted and 25 ml of 0.2 M NaCl was added; the pH was adjusted to 4.5 with 0.1–0.5 M NaOH. The enzyme was added (0.5 ml containing 205 units PG II; a unit of PG II releases 1 μmole of reducing groups from PGA in 30 min at pH 4.5 and 37°), soln-cell wall mixtures were vortexed and the time 0 treatment immersed in a boiling H_2O bath for 10 min. At each time, PG II was heat inactivated and the solns saved for soluble uronide determination [25]. Upon cooling, the treated wall material was dehydrated in EtOH, critical point dried and stored *in vacuo* as previously described.

General ^{13}C *CPMAS.* NMR spectra (8 kHz spectral width) were obtained at a ^{13}C frequency of 15 MHz; the ^1H decoupling field strength was 11 G. One K data points were sampled and zero filled to 4 K for data acquisition. All chemical shifts were assigned relative to hexamethylbenzene (HMB) methyl resonance ($\delta 17.36$) based on the position of TMS. Samples (300–500 mg dry wt) were spun at *ca* 2.4 kHz at the magic angle (54.7°) in a Kel-F bullet-type rotor. Setting the angle with HMB was performed prior to each relaxation expt. All ^{13}C CPMAS NMR expts were performed in the presence of dry N_2 flow. Areas under resonance peaks were determined by triangulating to the baseline and taking three planimeter measurements/area. Spectra used for quantitative measurements were acquired using 0.5 and 0.8 msec contact times. These times gave maximal signals for anomeric

*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

(δ105) and carbonyl (δ172) resonances, respectively. Areas under the methoxy resonance (δ54) were the same at either contact time.

CPMAS relaxation times ($^{13}C\ T_{1\rho}$, $^{1}H\ T_{1\rho}$, $^{1}H\ T_1$). Peak intensity measurements were used for the calculation of relaxation times. *Ca* 20 Hz computer line broadening was used for all frequency domain spectra. Values of $^{1}H\ T_1$ were determined indirectly via observation of ^{13}C magnetization after cross polarization in a 180°-t-90° pulse sequence (t = variable time delay). A contact time of 0.8 ms was used in all relaxation time measurements. Recycling times were *ca* $6 \times {}^{1}H\ T_1$s. In the inversion recovery expts, all signal intensities (S) were divided by the signal intensity observed after long time delays ($S_{t\infty} = S$ at 1 sec). These normalized signal intensities were then recalculated as $\{\ln[0.5\,(1\text{-}[S/S_{t\infty}])]\}$ and subjected to linear regression analysis as a function of the appropriate variable time delay. The slope of the calculated relationship $= -(1/T_1)$. ^{1}H or $^{13}C\ T_{1\rho}$ data were normalized to the greatest signal intensity (S_0), ln (S/S_0) was calculated, and the data were analysed via linear regression as a function of the time variable (contact time or ^{13}C spin lock time, respectively). The slope of the calculated linear relationship $= -(1/T_{1\rho})$. Slope standard errors (s.e.) were calculated for each measurement.

$^{23}Na^+$ *NMR*. Spectra (±25 kHz sweep width) were obtained at 500 MHz (^{1}H frequency). Samples were spun at *ca* 3.7 kHz at the magic angle. Peak intensity (100 Hz line broadening) measurements were used for the calculation of $^{23}Na^+\ T_1$s. The pulse sequence consisted of a train of ten 90° pulses separated with delays of 10 μsec followed by a variable delay (0.05–50 msec) during which T_1 relaxation would occur. After the variable delay, another 90° pulse was applied followed by data acquisition. T_1 calculations were performed as previously described ($S_{t\infty}$ = intensity at 50 msec).

Atomic absorption spectrophotometry. Ca^{2+} and Na^+ concns were determined by standard means.

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